Table 1
Baseline characteristics of patients in the before HAART and after HAART groups.

Characteristic	Before HAART group (n = 639)	After HAART group (n = 409) [a]
Age [median (IQR)]	32 (29–37)	33 (30–38)
Male gender [n (%)]	267 (41.8)	184 (45.0)
Clinical status [n (%)]		
Asymptomatic	334 (52.3)*	144 (35.2)*
Non-AIDS symptomatic	115 (18.0)	79 (19.3)
AIDS symptomatic	190 (29.7)*	186 (45.5)*
Previous ART [n (%)] [b]	148 (23.2)	101 (24.7)
Baseline CD4 cell count (cells/μl) [median (IQR)]	152 (25–348)*	44 (15–110)*
Baseline viral load (copies/ml) [median (IQR)] [c]	153 448 (33 058–5 189 295)	187 577 (62 791–490 282)
Previous OIs [n (%)]		
Tuberculosis [d]	71 (11.1)	53 (13.0)
Pneumocystis pneumonia	60 (9.4)	61 (14.9)*
Cryptococcal meningitis	51 (8.0)	49 (12.0)
Penicilliosis	19 (3.0)	35 (8.6)
Oesophageal candidiasis	8 (1.3)	47 (11.5)*
Herpes zoster	80 (12.5)	51 (12.5)
Toxoplasma encephalitis	18 (2.8)	8 (2.0)
Cytomegalovirus retinitis	12 (1.9)	25 (6.1)*

HAART: highly active antiretroviral therapy; IQR: interquartile range; ART: antiretroviral therapy; OIs: opportunistic infections.
[a] After HAART group include new patients as well as those who were enrolled in the before HAART group and who then started HAART.
[b] Experience with ART is limited to mono or dual therapy.
[c] Viral load data were available for only 274 patients in the after HAART group.
[d] Tuberculosis includes both pulmonary and extrapulmonary tuberculosis.
* Statistically significant (P < 0.05).

Results were presented as hazard ratios with 95% CIs. Statistical analyses were conducted using STATA version 10.0 (StataCorp LP, College Station, TX, USA).

3. Results

3.1. Patient characteristics

Of 756 HIV-infected persons recruited before GPO-Vir was introduced, 36 self-funded patients who were receiving HAART before recruitment were excluded. In addition, 16 patients who visited only once at enrolment and who died shortly after were also excluded from the analysis. Follow-up data on OIs were available for 639 (90.8%) of the remaining 704 patients. Total follow-up time was 1024.5 person-years of observation (PYO), with a median follow up of 476 days [interquartile range (IQR) 195–917 days]. During the observation period, 263 patients (41.2%) died, resulting in a mortality rate of 25.7/100 PYO (95% CI 22.6–28.8/100 PYO) in this group. In patients receiving HAART, the total duration of follow-up was 696.7 PYO from the time of treatment initiation, with a median follow-up duration of 720 days (IQR 677–722 days). During the observation period 32 patients died, resulting in a mortality rate of 4.6/100 PYO (95% CI 3.00–6.58/100 PYO), as described previously.[18]

The assumed transmission route was heterosexual in the majority of study patients (95%), with no change over time. Table 1 summarises the baseline characteristics of patients not receiving and receiving HAART. Demographic characteristics such as age and sex were similar. The proportion of patients who had previously received mono or dual ART was also similar between the groups. Patients in the HAART group were more likely to have AIDS or HIV-related symptoms and had a much lower CD4 cell count at enrolment or treatment initiation. Previous OIs (before enrolment) tended to be more common in patients receiving HAART.

3.2. Incidence rate of opportunistic infections and impact of HAART

Table 2 shows incidence rates and hazard ratios of different OIs according to HAART treatment status. In the before HAART group, TB was the most common OI, followed by PCP, cryptococcal meningitis and penicilliosis. In the HAART group, TB and cryptococcal meningitis were the two most common OIs, followed by penicilliosis. PCP was rare in patients on HAART. The incidence of cytomegalovirus (CMV) retinitis remained approximately stable after the introduction of HAART, but numbers were low. In univariate Cox regression analysis, all OIs combined decreased by 60%.

Multivariate Cox regression analysis revealed a great benefit of HAART (Table 2). After adjustment for baseline CD4 cell count, HAART reduced the incidence rate of all OIs by 80%. Further adjustment for age, gender, previous ART and AIDS-related symptoms in the full model had little additional effect on the hazard ratios (Table 2). The reduction in the incidence rate appeared to vary between OIs. The reduction in PCP incidence with HAART was the most substantial, with the reduction in TB and cryptococcal meningitis being significantly lower (P < 0.05). Exclusion of those patients from the HAART group who were enrolled before HAART was available and then went on to receive HAART (n = 195) did not result in marked changes in the hazard ratios.

Approximately 50% of cases of cryptococcal meningitis (8/15; 53.3%) and CMV retinitis (5/11; 45.5%) occurred within the first 2 months after the initiation of HAART with GPO-Vir. Approximately 90% of TB and herpes zoster cases occurred within 1 year, with a median of 175 days (range

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Table 2
Incidence of opportunistic infections among HIV-infected patients in the before HAART and after HAART groups.

Opportunistic infection	Before HAART group (n = 639)		After HAART group (n = 409)		HR (95% CI) [a]	aHR (95% CI) [b]
	Frequency	Incidence rate/100 PYO (95% CI)	Frequency	Incidence rate/100 PYO (95% CI)		
Tuberculosis [c]	59	5.9 (4.4–7.4)	17	2.5 (1.3–3.7)	0.4 (0.2–0.8)	0.2 (0.1–0.5)
Pneumocystis pneumonia	47	4.7 (3.3–6.0)	2	0.3 (−0.1 to 0.7)	0.06 (0.01–0.2)	0.03 (0.007–0.1)
Cryptococcal meningitis	41	4.2 (2.9–5.5)	15	2.2 (1.1–3.3)	0.5 (0.3–0.9)	0.2 (0.1–0.5)
Penicilliosis	35	3.5 (2.3–4.6)	9	1.3 (0.5–2.2)	0.4 (0.2–0.8)	0.1 (0.06–0.3)
Oesophageal candidiasis	19	1.9 (1.0–2.7)	3	0.4 (−0.1 to 0.9)	0.2 (0.06–0.8)	0.1 (0.02–0.5)
Herpes zoster	40	4.0 (2.7–5.2)	13	1.9 (0.9–3.0)	0.6 (0.3–1.1)	0.5 (0.2–1.0)
Toxoplasma encephalitis	12	1.2 (0.5–1.9)	7	1.0 (0.3–1.8)	0.8 (0.3–2.1)	0.4 (0.1–1.5)
Cytomegalovirus retinitis	18	1.8 (1.0–2.6)	11	1.6 (0.7–2.6)	1.0 (0.4–2.4)	0.6 (0.2–1.7)
All AIDS-defining illnesses	180	19.1 (16.3–21.9)	53	8.2 (6.0–10.4)	0.4 (0.3–0.6)	0.2 (0.1–0.3)

HAART: highly active antiretroviral therapy; PYO: person-years of observation; HR: hazard ratio; aHR: adjusted hazard ratio.
[a] Used robust standard errors.
[b] Adjusted by baseline CD4 cell count, gender, age, AIDS-related symptoms and antiretroviral therapy history.
[c] Includes both pulmonary and extrapulmonary tuberculosis.

51–532 days; IQR 100–274 days) for TB and 125 days (range 9–615 days; IQR 109–195 days) for herpes zoster. In the 189 patients who developed OIs before HAART was available 49 patients (25.9%) experienced more than one OI, whereas in the 53 patients who developed OIs in the HAART group only 6 (11.3%) were diagnosed with multiple OIs.

3.3. *Opportunistic infection-free survival curves*

Figure 1 shows Kaplan–Meier OI-free survival curves for the four most common OIs (TB, PCP, cryptococcal meningitis and penicilliosis) for patients not receiving and receiving HAART, stratified by baseline CD4 cell count. Among the patients in the before HAART group, as expected a lower CD4 cell count at baseline was strongly associated with the development of OIs, except for TB for which the disease-free survival curves overlapped in the lowest and middle CD4 strata. All OIs were rare in the higher CD4 stratum.

In the highest CD4 stratum of patients on HAART, none of the patients developed any of the four OIs. CD4 cell count was associated with TB and cryptococcal meningitis. PCP and penicilliosis were rare in all CD4 count strata.

4. Discussion

This study demonstrates a substantial reduction in the incidence of major OIs following the introduction of HAART at a government referral hospital in northern Thailand.

Compared with reports from high-income countries, it was found that the incidence of OIs was higher both before and after starting HAART. The Swiss HIV Cohort Study reported an overall incidence of AIDS-related OIs within 6 months before and within 15 months of starting HAART of 15.1 and 3.6 incidence per 100 PYO, respectively.[19] The incidence of OIs even after introduction of HAART was twice as high in Thailand than in Europe. We believe that one of the reasons for the high incidence of OIs in this cohort was the low baseline CD4 cell count, as this is one of the strongest risk factor for OIs according to our results and studies elsewhere.[20,21] Another explanation might be the high incidences of TB, cryptococcal meningitis and penicilliosis. Whilst none of these OIs are common in high-income countries, TB is the most common OI and also the leading cause of mortality in HIV-infected patients in resource-limited settings.[22,23] In a cohort study focused on TB in South Africa, the overall incidence of TB among HIV-infected individuals on HAART was 2.4/100 PYO, similar to the current cohort,[13] although the TB incidence in the present cohort might have been particularly high since the median CD4 cell count was lower than in the South Africa cohort.

In resource-limited settings, most patients often present late to ART programmes, with low median CD4 cell counts, a high risk of new HIV-related diseases and high early mortality. During the first year of study, between 8% and 26% of patients have been shown to die during the first year of HAART, with most deaths occurring during the first few months.[6] TB and cryptococcal meningitis are leading causes of early mortality, accounting for up to 20% of all deaths[24] in high HIV prevalence regions.

Previous studies on the incidence of penicilliosis or cryptococcal meningitis in developing countries are scarce.[25,26] The high incidence of fungal OIs such as cryptococcal meningitis and penicilliosis in the present cohort appears to be typical for northern Thailand, southern China and northern Vietnam.[21] In contrast, MAC infection and Kaposi sarcoma, which were reported to be relatively common in high-income countries, were not common in this study.[19] Kaposi sarcoma is known to be rare in Thailand;[27] in fact, no case of Kaposi sarcoma has been diagnosed since the DCC of Lampang Hospital was established 1995. The prevalence of human herpes virus type 8 (HHV8) may be lower among the heterosexual population in northern Thailand. MAC infection may be underdiagnosed due to the difficulty of confirming the pathogen in blood culture.

Similar to previous reports from the USA, Canada and Europe,[1,19,28] there was a major decline in the incidence of almost every OI following initiation of HAART in the present study. We are unaware of studies from middle- or low-income countries evaluating the impact of HAART on individual OIs other than TB with which our results can be compared.[14,29] In the present study, reduction in the incidence of TB was consistent with a report from South Africa.[13] Some evidence that the effect of HAART on PCP was stronger than the effect on TB was also found; TB and cryptococcal meningitis remained quite common after the

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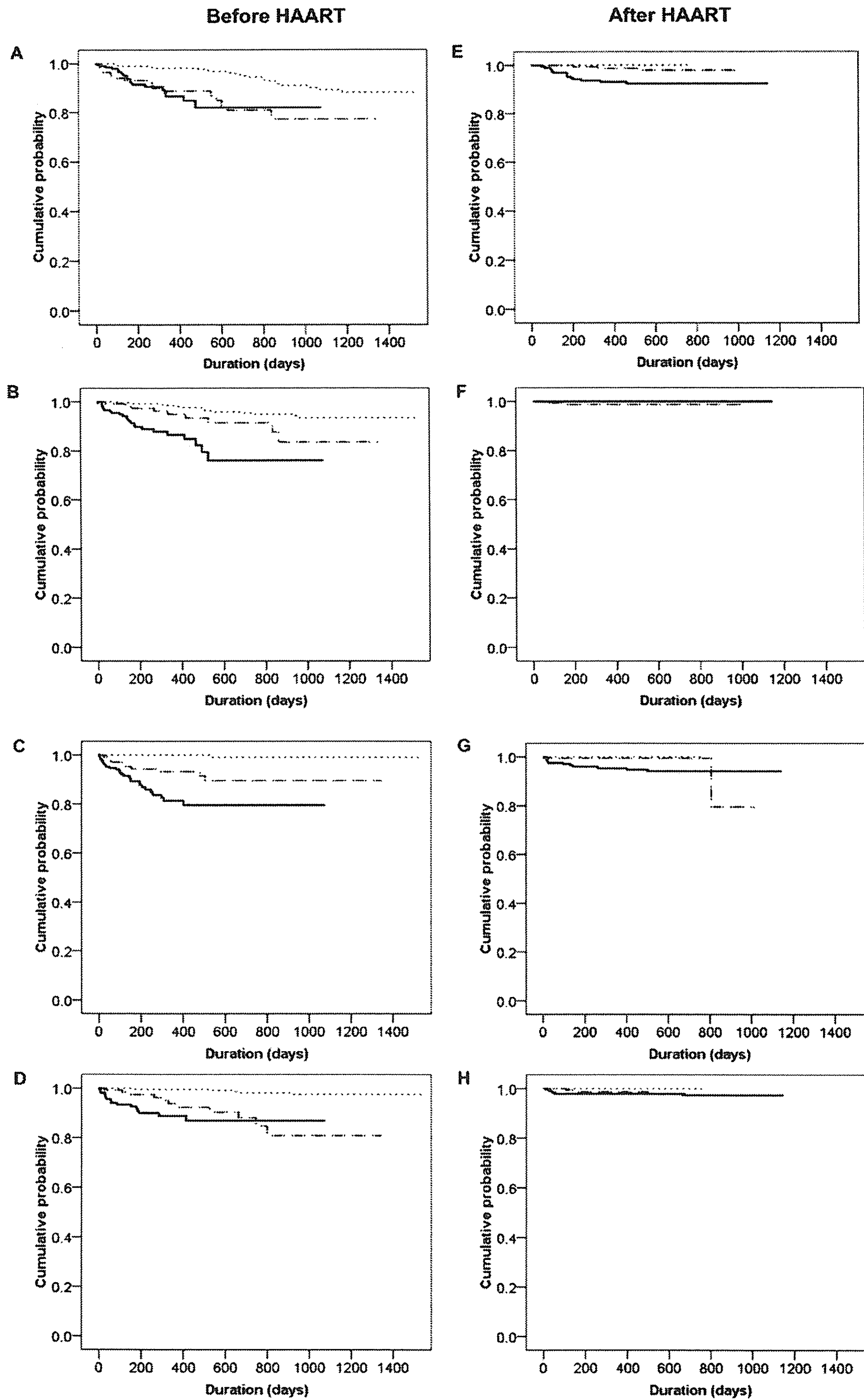

Figure 1. Kaplan–Maier estimates of patients diagnosed with major opportunistic infections before and after highly active antiretroviral drug therapy (HAART) stratified by baseline CD4 cell count: solid thick line, CD4 cell count < 50 cells/μl; dashed line, 50–199 cells/μl; and dotted line, ≥200 cells/μl. Cumulative probability indicates patients free from tuberculosis (A and E), pneumocystis pneumonia (B and F), cryptococcal meningitis (C and G) and penicilliosis (D and H).

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initiation of HAART, although the majority of such cases occurred within 1 year after starting HAART.

In contrast to TB and cryptococcal meningitis, PCP almost disappeared. Primary prophylaxis might have been more effective against PCP compared with prophylaxis against cryptococcal meningitis and toxoplasmosis. However, the same practice was applied for patients before receiving HAART. The Swiss HIV Cohort Study also showed that the decline in incidence was most pronounced for Kaposi sarcoma, followed by PCP.[19] In the current study, only marginal change in the incidence of CMV retinitis before and after HAART was noted, possibly because CMV retinitis before HAART tended to be underdiagnosed. Because of the limited availability of ophthalmologists in most government HIV clinics, ophthalmological screening is not routine practice. One-half of CMV cases were diagnosed shortly after starting HAART as they developed visual symptoms, which might be due to the immune reconstitution inflammatory syndrome (IRIS).[30] CMV infection is known to be one of the most common OIs associated with IRIS.[31] The relatively high incidence of CMV retinitis in the current cohort, recognised shortly after the initiation of HAART, might therefore be linked to IRIS.

This study is limited by the before/after design without a concurrent control group as in a randomised controlled trial. Patients enrolled after the introduction of HAART tended to have more unfavourable clinical characteristics, which may contribute to understating the effect of HAART. The effect size was therefore adjusted for the CD4 cell count as the most important predictor of OI. Additional adjustment for other potential confounders had little effect on the hazard ratios, but some residual confounding may still be present.

In the present study, the before and after HAART groups were followed-up at the same clinic, and throughout the observation period only three clinicians were involved in the management of patients. Knowledge of treatment allocation by clinicians assessing clinical symptoms may lead to bias towards a greater impact of HAART because of doctors' expectations as to its effectiveness. On the other hand, improved treatment options for a previously fatal disease can raise the motivation of staff to diagnose OIs more accurately, biasing the effect of HAART toward null. To minimise the risk of observer bias, the clinicians followed a local standardised guideline developed to suit the management of HIV patients in Lampang Hospital.

In summary, a substantial reduction in incidence of individual OIs was seen after starting ART in this setting. PCP almost disappeared among patients on GPO-Vir, whereas TB and cryptococcal meningitis remained relatively common OIs especially within the first year of starting HAART. In light of these findings, chemoprophylaxis, screening, and early diagnosis and treatment for TB and cryptococcal meningitis deserve attention in the HAART era among HIV patients with low CD4 cell counts in resource-limited settings, especially in northern Thailand.

Authors' contributions: AR, PP, NT, WA, PS and KA conceived the study and designed the study protocol; AR, WP and PP carried out the clinical assessment and management; PS and WA helped with organisation and execution of the study; NT contributed to data collection; AR, NT and KA analysed the data; all authors contributed to interpretation of the data; AR, NT and KA drafted the manuscript; W-PS and SH provided statistical support. All authors read and revised the manuscript critically for intellectual content. All authors approved the final manuscript. KA is guarantor of the paper.

Acknowledgements: The authors thank the Department of Medical Sciences of the Ministry of Public Health of Thailand; all of the patients enrolled in the study; and Dr Somsak Thamthitiwat and the staff at Lampang Hospital, especially Ms S. Kasemsuk, Ms S. Seneewong-naayudhaya, Ms A. Suyasarojna, Mr P. Wongnamnong, Ms K. Yod-dumnern, Ms K. Lor-yont, Mr W. Khaewkarnka, Mr S. Umnajsirisuk and Mr. S Niyom-thai.

Funding: Japan International Co-operation Agency (JICA), Thai Ministry of Public Health and Japan Ministry of Education, Culture, Sports, Science and Technology grant-in-aids.

Conflicts of interest: None declared.

Ethical approval: This study was conducted as part of the Lampang HIV Cohort Phase I and the Lampang and Phayao HIV Cohort Phase II, which were approved by Thai Ministry of Public Health's Research Ethics Committee. Written informed consent was obtained from all participants upon enrolment.

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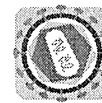

RETROVIROLOGY

SHORT REPORT | Open Access

Effects of naturally-arising HIV Nef mutations on cytotoxic T lymphocyte recognition and Nef's functionality in primary macrophages

Philip Mwimanzi, Zafrul Hasan, Ranya Hassan, Shinya Suzu, Masafumi Takiguchi and Takamasa Ueno*

Abstract

Background: Although HIV can infect several cellular subsets, such as $CD4^+$ T lymphocytes and macrophages, it remains unclear whether an HIV infection in macrophages supports cytotoxic T lymphocyte (CTL) escape. Here, we tested two naturally-arising mutations located in the well-conserved polyproline region of Nef for their effects on CTL recognition, Nef's functionality, and viral replication capacity in macrophages. These mutations were selected because they are known to cause CTL escape in the context of T lymphocytes.

Findings: Monocyte-derived macrophages (MDMs) infected with the wild-type virus, but not with variant viruses, were efficiently killed by CTL clones targeting Nef epitopes, VY8 (VPLRPMTY) and RY11 (RPQVPLRPMTY). The CTL-escape mutation, $Arg^{75}Thr$, or $Arg^{75}Thr/Tyr^{85}Phe$ double mutation, reduced the HLA class I down-regulation activity and, interestingly, increased the susceptibility of virus-infected MDMs to recognition by CTLs targeting a different epitope. The same mutations reduced the CCR5, but not CD4, down-regulation activity. Moreover, the Nef variants were impaired for Hck activation and enhancement of viral replication in MDMs.

Conclusions: These results suggest that HIV-infected MDMs are killed by CTLs targeting Nef epitopes, contributing to selection and adaptation of CTL-escape viral variants.

Findings

Several different cellular subsets such as $CD4^+$ T lymphocytes, macrophages, and dendritic cells can be targets for an HIV infection; although they differentially support HIV replication and persistence *in vivo* [1-3]. Macrophages may be the early target of HIV, but are highly resistant to the cytopathic effects of an HIV infection and continuously produce infectious virions for a long period of time [4,5]. It is thought that the differences in fitness of viral replication among the different cellular environments could influence the selection and adaptation of viral quasispecies in these cells. The HLA class I-restricted $CD8^+$ cytotoxic T lymphocyte (CTL) response is thought to play an important role in controlling HIV replication [6-8] and to mediate a major selective force for the emergence of viral variants [9,10]. Certain CTL escape mutations, in well-conserved regions of Gag and Nef, have been reported to impose functional constraints on these proteins and to modulate viral replication in the context of T lymphocytes [11-13]. However, in the context of macrophages, the selection of CTL escape variants and functional adaptation of viral proteins are not yet fully understood. We previously showed that the HLA-B35-restricted CTL responses toward a well-conserved proline-rich region in Nef results in the emergence of a CTL escape mutation, either Arg75Thr or Tyr85Phe, from phylogenetically different viral quasispecies even within an HIV-infected host [13]. These mutations constrain some of the important Nef functions in $CD4^+$ T cells [13]. Here we tested whether an HIV-1 infection in macrophages would have any influence on CTL recognition and escape as well as Nef's functionality and adaptation in the infected macrophages.

Susceptibility of HIV-infected macrophages to recognition by the cognate CTLs

We previously reported that in HIV-infected patients with HLA-B35, the Nef protein elicits dominant CD8 T

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cell responses [14], with the short epitope VY8 (Nef_{78-85}; VPLRPMTY) being the early epitope, which subsequently shifts to the amino terminal-extended longer epitope RY11 (Nef_{75-85}; RPQVPLRPMTY) [13]. Autologous virus sequence analysis revealed that the mutations Tyr85 to Phe (85F) and Arg75 to Thr (75T) are associated with the early and chronic phase of an HIV infection, respectively, in HIV-infected individuals with *HLA-B35* but that these 85F and 75T mutations are derived from phylogenetically different lineages [13].

We first examined CTL activity toward macrophages infected with HIV-1 strain JRFL, in which *nef* gene had been replaced with that of strain SF2 (referred as JRFL-SF2nef) and its variants. In this JRFL-SF2nef, we had created unique restriction sites, *Cla* I and *Not* I adjacent to the ends of the *nef* open reading frame [15] and confirmed that the resultant viruses, prepared by transfecting 293 T cells with JRFL and JRFL-SF2nef, had comparable replication capacity in primary monocyte-derived macrophages (MDMs) (data not shown). To prepare mature MDMs, $CD14^+$ cells were isolated from PBMCs of HIV-negative donors, in accordance with the human experimentation guidelines of Kumamoto University, and cultured for 7 days in the presence of 100 ng/ml of macrophage colony-stimulating factor (Peprotech GmbH, Germany). Previously established CTLs, specific for VY8 and RY11, [13,14] were highly cytotoxic toward MDMs infected with wild-type (wt) HIV-1, suggesting that HIV-infected MDMs were a preferable target for CTLs. The VY8-specific CTLs showed higher cytotoxicity toward wt virus-infected MDMs than did the RY11-specific CTLs (Figure 1), in good agreement with the observation obtained with HIV-infected $CD4^+$ T cells [13,16]. In contrast, VY8- and RY11-specific CTLs failed to kill primary MDMs infected with 85F and 75T viruses, respectively (Figure 1), indicating that the 85F and 75T single mutations conferred escape from CTLs specific for VY8 and RY11, respectively, but not simultaneously. In contrast, the TF virus could escape from both types of CTLs (Figure 1). It should be noted that Western blot analysis of Nef proteins in virus-producing cells showed a comparable level of Nef expression among wt and all variant viruses except for ΔNef (data not shown).

Effects of the Nef mutations on Hck activation

Nef is known to associate via its PxxP motif with the SH3 domain of several different cellular kinases including Hck [17,18]. We tested whether the CTL-escape variants in the PxxP region would affect the Hck activation by Nef by using the *in vitro* Hck activation assay as described earlier [19] (Figure 2A). Expectedly, the wild-type Nef showed robust Hck activation; whereas the AxxA variant Nef (Pro76Ala and Pro79Ala) did not show substantial activation (Figure 2B). The 85F variant Nef did not affect Hck activation, whereas the Hck activation was substantially reduced by the 75T and TF variants of Nef (Figure 2B). These results suggest that CTL-escape variants in the PxxP motif affect Hck activation in macrophages.

Effects of the Nef mutations on HLA class I down-regulation

Because Nef helps HIV-infected cells to evade CTL lysis by down-modulating cell-surface HLA-I and the PxxP motif is critical for this activity [13,20,21], we examined the HLA-I down-regulation activity by Nef in MDMs infected with wt and variant viruses by flow cytometry (Figure 3A). The surface levels of HLA-I within $p24^+$ subsets in wt virus-infected MDMs were much reduced compared with those in uninfected cells (Figure 3B) and that no HLA-I down-regulation was observed in ΔNef virus-infected MDMs (Figure 3B). In contrast, both the 75T and the TF variant viruses showed substantially diminished down-regulation activity; whereas the 85F variant virus showed down-regulation activity comparable to that of the wt (Figure 3B).

Susceptibility of HIV-infected MDMs to recognition by CTLs of another specificity

We postulated that the impaired Nef-mediated down-regulation activity of HLA-I in MDMs could influence the susceptibility to killing of HIV-infected MDMs by CTLs. To test this, we first created the variant virus having M20A or P82A (numbering based on the SF2 strain) because these mutations have been shown to completely disrupt the Nef-mediated HLA-I down-regulation activity [22,23]. We then assessed the cytolytic activity of CTL clones specific for another Nef epitope presented by HLA-A24 ($Nef_{138-147}$: RYPLTFGWCF) toward MDMs infected with wt, M20A, or P82A viruses. Although the amino-acid sequences in the epitope region of A24-Nef were the same among the wt and these variant viruses tested, the CTL-mediated killing activity toward MDMs infected with M20A and P82A variant viruses was much increased compared to those infected with the wt virus (Figure 4).

Next, we also determined CTL cytotoxic activity toward MDMs infected with 75T, 85F, and TF variant viruses. The A24-Nef CTLs showed the most potent activity toward MDMs infected with either the 75T or TF variant viruses; whereas their cytotoxic activity was less potent toward MDMs infected with either the wt or the 85F mutant virus (Figure 4). These data suggest that the diminished HLA-I down-regulation (i.e., increased level of cell-surface HLA-I) in MDMs infected with the 75T and the TF mutant viruses (Figure 3) resulted in increased susceptibility to killing by CTLs of another specificity (Figure 4), leading to a possible

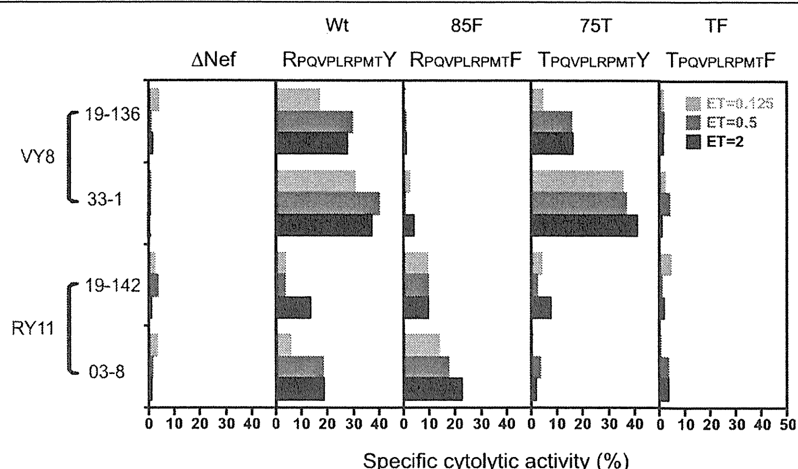

Figure 1 Susceptibility of HIV-infected MDMs to recognition by the cognate CTLs. Cytotoxic activity of HLA-B35-restricted CTL clones specific for VY8 (VPLRPMTY) and RY11 (RPQVPLRPMTY) epitopes in Nef toward HIV-infected MDMs is shown. MDMs were isolated from an HIV-negative donor (*HLA-B*35:01*⁺) and infected with wild-type or one of the variant viruses indicated. The resultant HIV-infected MDMs (2×10^3/well) were then mixed with CTL clones at various effector-to-target cell ratios (E/T) for 6 hr at 37°C after having been labeled with ^{51}Cr. The frequency of HIV-infected cells among target cells as determined by intracellular p24 Ag expression was 48.7, 55.4, 51.0, and 48.8% for wt, 85F, 75T, and TF variants, respectively. CTL 19-136 and 19-142 were derived from the same HIV-infected donor (019), and CTL 33-1 and 03-8 were derived from different donors, 033 and 03, respectively. CTL activity toward uninfected cells was deducted from the data as background. An additional experiment showed similar results.

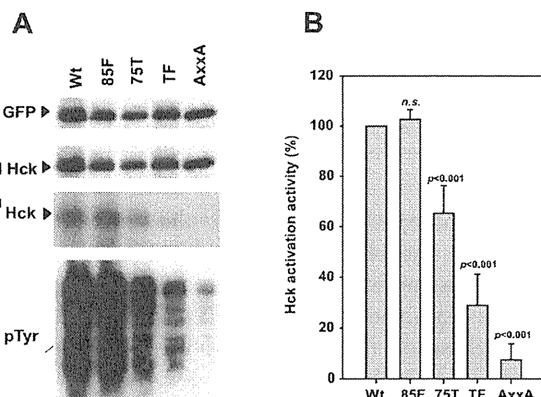

Figure 2 Hck activation. (A) HEK293 cells were transfected with cDNA encoding GFP alone or Nef-GFP fusion proteins in the presence of Hck plasmid, and analyzed by Western blotting with anti-GFP (FL; Santa Cruz), anti-Hck (clone 18; Transduction Laboratories, Lexington, KY), anti-Hck phosphorylated at tyrosine 411 (Hck-pTyr411; Santa Cruz) and anti-phosphotyrosine (PY99; Santa Cruz) as indicated. The Nef variants tested are indicated in the figure. A representative datum set of 3 independent experiments is shown. (B) Quantification of Hck activation by Nef. The indicated values represent the Hck activation activity after the level of phosphorylated Hck had been normalized to the amounts of total Hck. The values presented were calculated from the data shown in panel A, and are relative to the wild-type control arbitrarily set to 100%. Data represent the means ± SD of 3 independent experiments, and statistical analysis was performed based on ANOVA with multiple comparisons *vs.* wt (Bonferroni *t*-test). *n.s.*, not significant.

selective disadvantage for the variant viruses under anti-HIV CTL responses.

Effects of the Nef mutations on down-regulation of viral receptors

We also examined whether Nef's down-regulation activity of viral receptors, i.e., CD4 and CCR5, could be influenced by the mutations in HIV-infected MDMs (Figure 5A). The cell-surface expression of CCR5 was substantially reduced in wt virus-infected MDMs but not affected in the ΔNef variant virus-infected ones (Figure 5B). Interestingly, the 85F variant virus showed CCR5 down-regulation activity comparable to that of the wt virus; whereas the 75T and TF variant were substantially impaired in this activity in MDMs (Figure 5B). In contrast, CD4 down-regulation activity was not affected for all of the viruses with mutated Nefs except for ΔNef (Figure 5C), consistent with the observation that CD4 down-regulation activity is mediated by a specific region in Nef other than the PxxP motif [21].

Effects of the Nef mutations on viral replication

We finally examined whether the mutations would differently affect the enhancement of viral replication in MDMs. In MDMs from 2 HIV-negative donors, the wt HIV-1 showed the highest replication among the various viruses tested; whereas the ΔNef variant showed much decreased replication (Figure 6A), consistent with the

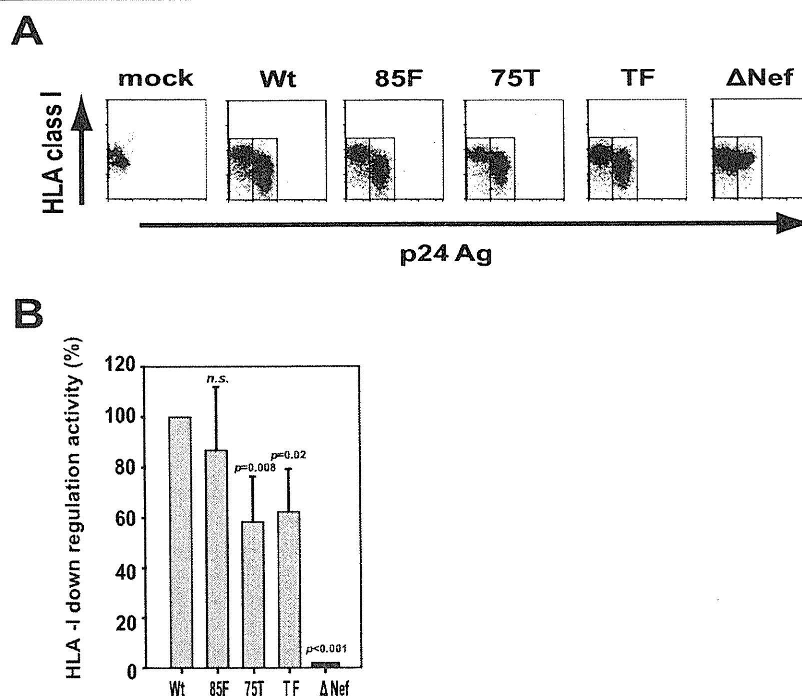

Figure 3 HLA class I down-regulation in HIV-infected MDMs. (A) MDMs prepared from an HIV-negative donor were infected with wild-type or variant viruses as indicated. Cells were stained with 7-amino-actinomycin D (7-AAD; BD Biosciences, CA) and anti-HLA class I allotype antibody SFR8-B6 followed by intracellular staining with FITC-labeled anti-p24 Gag mAb (KC-57; Beckman Coulter, CA) as described before [13]. In flow cytometric analysis (FACS Canto II, BD Biosciences), cells negative for 7-AAD were gated and analyzed for their fluorescence intensity for HLA class I and p24 Gag. (B) The same experiment as above was done by using 3 additional HIV-negative donors. The HLA class I allotype-specific antibodies used were either SFR8-B6 or A11,1 M as appropriate for each donor. The relative down-regulation activity of HLA class I by wt Nef and its variants is presented relative to that of the wild-type Nef activity set to 100%. Data represent the means ± SD of all 4 donors, and statistical analysis was performed based on ANOVA with multiple comparisons *vs.* wt (Bonferroni *t*-test). *n.s.*, not significant.

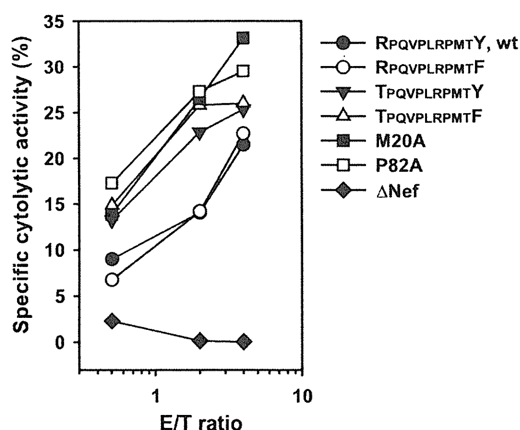

Figure 4 Susceptibility of HIV-infected MDMs to recognition by CTLs of another specificity. MDMs prepared from an HIV-negative donor (*HLA-A*24:02*+) infected with the indicated viruses were used as target cells for cytolysis by an HLA-A24-restricted CTL clone specific for the Nef epitope ($Nef_{138-147}$: RYPLTFGWCF). The frequency of HIV-infected cells among the target cells, as determined by intracellular p24 Ag expression, was 41.4, 48.3, 44.5, 40.8, 40.0, and 45.0% for wt, 85F, 75T, TF, M20A, and P82A variants, respectively. CTL activity toward uninfected cells was deducted from the data as background. An additional experiment showed similar results.

previous observation [24]. The replication of the 85F variant virus was partially impaired in MDMs from one of the donors and was comparable to that of the wt virus in MDMs from the other donor (Figure 6A). In contrast, the replication of the 75T and TF variant viruses was impaired in MDMs from both donors (Figure 6A). To account for this donor variability, we summarized the results from a total of 5 donors in Figure 6B. Because the peak of the virus replication was between 6 to 12 days after infection, depending on the donor and the virus, the peak p24 Ag values of each of the viruses are presented and were used for statistical analysis (Figure 6B). The 75T and the TF variant viruses showed significantly diminished capacity for viral replication compared with the wt; whereas the 85F virus did not show much difference in replication capacity (Figure 6B).

Discussion and Conclusions

Although the Nef protein is thought to have very high mutational plasticity, we showed here that the naturally-arising CTL escape variants in the well-conserved PxxP region in Nef alone or in combination can modulate some pathogenic functions of Nef in the

Figure 5 Viral receptor down-regulation in HIV-infected MDMs. (A) MDMs prepared from an HIV-negative donor were infected with wt or variant viruses as indicated. The cells were stained with 7-AAD and allophycocyanin-Cy7 anti-human CD4 mAb (Biolegend, CA) and phycoerythrin-Cy7-conjugated anti-human CCR5 mAb (BD Biosciences) followed by intracellular staining with antibody against p24 Gag. In flow cytometric analysis, cells negative for 7-AAD and positive for p24 Gag were gated and analyzed for their fluorescence intensity for CD4 and CCR5. (B, C) The same experiment as above was done by using 3 additional HIV-negative donors. The relative down-regulation activity of wt Nef and its various variants toward CCR5 (panel B) and CD4 (panel C) is presented, with the wt Nef activity set to 100%. Data represent the means ± SD of all 4 donors, and statistical analysis was performed based on ANOVA with multiple comparisons *vs.* wt (Bonferroni *t*-test). *n.s.*, not significant.

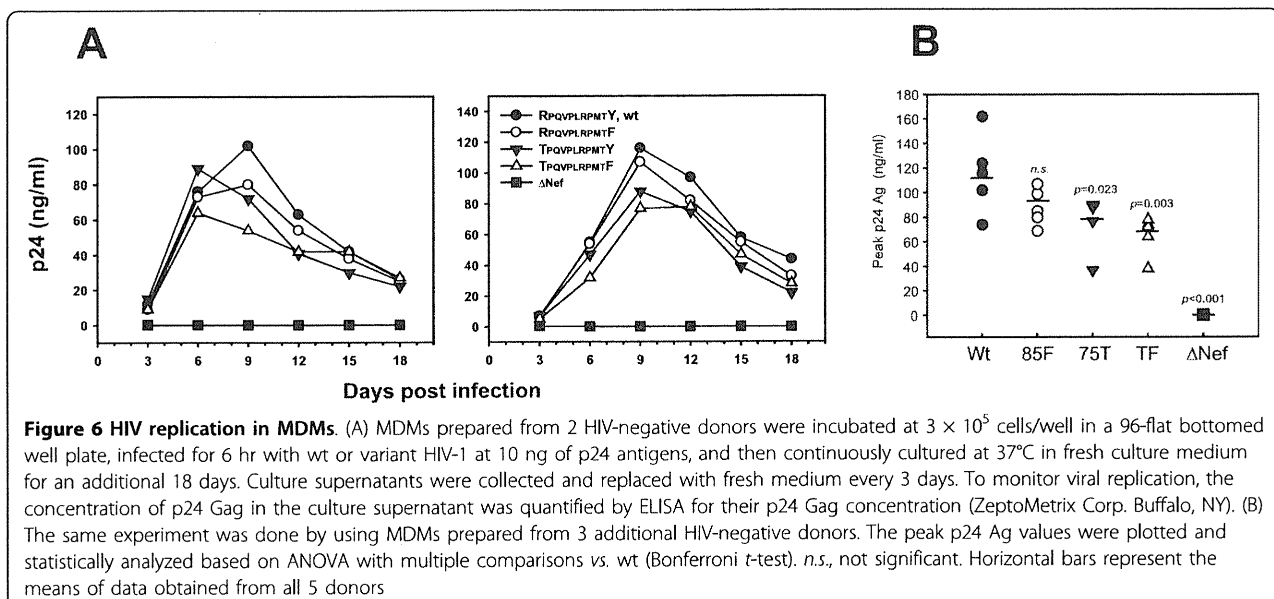

Figure 6 HIV replication in MDMs. (A) MDMs prepared from 2 HIV-negative donors were incubated at 3×10^5 cells/well in a 96-flat bottomed well plate, infected for 6 hr with wt or variant HIV-1 at 10 ng of p24 antigens, and then continuously cultured at 37°C in fresh culture medium for an additional 18 days. Culture supernatants were collected and replaced with fresh medium every 3 days. To monitor viral replication, the concentration of p24 Gag in the culture supernatant was quantified by ELISA for their p24 Gag concentration (ZeptoMetrix Corp. Buffalo, NY). (B) The same experiment was done by using MDMs prepared from 3 additional HIV-negative donors. The peak p24 Ag values were plotted and statistically analyzed based on ANOVA with multiple comparisons *vs.* wt (Bonferroni *t*-test). *n.s.*, not significant. Horizontal bars represent the means of data obtained from all 5 donors

context of human primary macrophages infected with a CCR5-tropic virus. There are 2 different aspects of CTL-mediated functional constraints on the PxxP-dependent Nef activities in MDMs reported here, one through immune evasion activity (HLA-I down-regulation activity) and the other acting on the intrinsic capacity to boost viral replication and persistence (Hck activation, viral co-receptor down-regulation activity, and enhancement of viral replication). In particular, one of the single mutants, 75T, impaired these Nef activities in MDMs infected with a CCR5-tropic virus. This is in line with the previous report showing that 75T mutation modulated Nef-stimulated viral replication in immature dendritic cell/T cell cocultures infected with a CCR5-tropic virus [25] although this mutation alone had virtually no influence on the same Nef activities in primary $CD4^+$ T cells infected with a CXCR4-tropic virus in the previous study [13]. In addition, the 75T mutation, located outside the VY8 epitope, reduced the cytolytic activity of VY8-specific CTLs in the context of $CD4^+$ T cells [13], but did not affect their cytolytic activity in the context of MDMs (Figure 1), suggesting the differential intracellular processing of the VY8 peptide between $CD4^+$ T cells and MDMs. This observation is in line with the previous report showing a substantial difference in intracellular processing of antigenic HIV peptides between monocytes and lymphocytes [26]. Overall, these results suggest that an antigenic variation of viruses can differentially influence viral replication and persistence between cellular subsets because of their different effects on the intracellular antigen-processing machinery, the susceptibility to CTL killing, as well as the fitness cost to viral replication.

Of particular interest are the data showing that the CTL-escape Nef mutation, 75T, impaired HLA-I down-regulation activity by Nef and rendered the HIV-infected MDMs more susceptible to killing by CTLs with another specificity. Such phenomenon was also observed in the context of $CD4^+$ T cells in our previous study [13]. However, wt-virus-infected cells, regardless of $CD4^+$ T cells or MDMs, could be killed to some extent by CTLs, suggesting that the Nef-mediated HLA-I down-regulation is insufficient for HIV to escape from CTL recognition and that, CTL-escape variant viruses are selected and emerged. Conversely, Swigut *et al.*, [27] reported that monkeys infected with SIV containing *nef* mutations that selectively eliminated MHC down-regulation activity exhibited higher level of SIV-specific CD8 T cell responses. In any event, an important question remains to be addressed which is how significant is Nef-mediated HLA-I down-regulation activity for HIV replication and persistence in HIV-infected humans.

Although HLA-B*35-restricted CTLs targeting PxxP region of Nef can impose functional constraints in viral replication in this study, we did not find any beneficial effects on clinical parameters (such as CD4 count and viral load) in HIV-infected patients with HLA-B*35 as well as those with HLA-B*35 and HLA-A*24 in our cohort to date (data not shown). Functional impairment in Nef induced by CTL-escape variants could be compensated later by mutations at secondary sites in Nef. For example, an inverse dose-response relationship has been observed between the number of CTL-escape mutations in Nef and CD4 counts in patients in a large population study [28]. Therefore, only some CTL-escape variants may play a role in modulating Nef functions *in vivo*, such as in the case of $HLA\text{-}B57^+$ elite suppressors [29]. Further studies using a large number of clinically-isolated *nef* alleles are needed to extend this observation, such as how Nef-specific CTL responses, Nef functions, and clinical outcome of HIV-infected individuals are related to each other at the population level.

Acknowledgements
We thank Dr. M Fujiwara and Ms. S. Doki for their great help. This research was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan and by a grant-in-aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan.

Authors' contributions
PM, MT, and TU designed the study. PM, ZH, RH, SS, and TU conducted the experiments. PM, SS, and TU wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 11 March 2011 Accepted: 22 June 2011
Published: 22 June 2011

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doi:10.1186/1742-4690-8-50
Cite this article as: Mwimanzi *et al.*: **Effects of naturally-arising HIV Nef mutations on cytotoxic T lymphocyte recognition and Nef's functionality in primary macrophages.** *Retrovirology* 2011 8:50.

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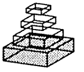
ORIGINAL RESEARCH ARTICLE
published: 10 January 2012
doi: 10.3389/fmicb.2011.00280



Fluorescent reporter signals, EGFP, and DsRed, encoded in HIV-1 facilitate the detection of productively infected cells and cell-associated viral replication levels

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Flow cytometric analysis is a reliable and convenient method for investigating molecules at the single cell level. Previously, recombinant human immunodeficiency virus type 1 (HIV-1) strains were constructed that express a fluorescent reporter, either enhanced green fluorescent protein, or DsRed, which allow the monitoring of HIV-1-infected cells by flow cytometry. The present study further investigated the potential of these recombinant viruses in terms of whether the HIV-1 fluorescent reporters would be helpful in evaluating viral replication based on fluorescence intensity. When primary CD4$^+$ T cells were infected with recombinant viruses, the fluorescent reporter intensity measured by flow cytometry was associated with the level of CD4 downmodulation and Gag p24 expression in infected cells. Interestingly, some HIV-1-infected cells, in which CD4 was only moderately downmodulated, were reporter-positive but Gag p24-negative. Furthermore, when the activation status of primary CD4$^+$ T cells was modulated by T cell receptor-mediated stimulation, we confirmed the preferential viral production upon strong stimulation and showed that the intensity of the fluorescent reporter within a proportion of HIV-1-infected cells was correlated with the viral replication level. These findings indicate that a fluorescent reporter encoded within HIV-1 is useful for the sensitive detection of productively infected cells at different stages of infection and for evaluating cell-associated viral replication at the single cell level.

Keywords: HIV-1, flow cytometry, EGFP, DsRed, Gag, productive infection

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) interacts with its primary receptor, CD4, and a co-receptor, usually CCR5 or CXCR4, to infect T cells, macrophages, and dendritic cells (Mcclure et al., 1987; Berger et al., 1999; Tsunetsugu-Yokota, 2008). Single cell analysis of HIV-1-infected cells is an essential approach to investigate the differential dynamics of HIV-1 infection and the cellular consequences for each of the HIV-1-targeted cell populations. To monitor HIV-1 infection, a recombinant HIV-1 encoding a reporter luciferase (Luc) gene, or indicator cells transduced with enzymatic reporters such as Luc, β-galactosidase, alkaline phosphatase, and chloramphenicol acetyl transferase, incorporated downstream of the HIV-1 long terminal repeats (LTR) have been widely used (Kar-Roy et al., 2000). However, these reporters require additional substrates or co-factors, and lysis or fixation of cells is required to show reporter activity, which makes the experimental process more complex. In addition, it is difficult to distinguish infected cells from uninfected cells using these reporter assays.

An alternative molecule, green fluorescent protein (GFP) and/or its derivatives, is a powerful reporter that does not require any substrates and co-factors to generate a reporter signal (Chalfie, 1995; Cubitt et al., 1995; Heim et al., 1995). Page et al. (1997) first used a GFP derivative, called enhanced green fluorescent protein (EGFP), as a fluorescent reporter molecule for HIV-1 and showed that infected cells were detectable and, more importantly, distinguishable from uninfected cells using flow cytometry. Furthermore, a red fluorescent protein, DsRed, has been used as an HIV-1 fluorescent reporter (Weber et al., 2006). The main benefit of such recombinant HIV-1 molecules is that the targeted cells do not require any modulation (e.g., transfection) of exogenous reporter genes and, therefore, they allow the characterization of intact HIV-1-infected cells. In most cases of previous recombinant HIV-1 strains, the *nef* gene was replaced with a reporter gene. Therefore, we previously constructed *nef*-intact, replication-competent, recombinant HIV-1 strains encoding either EGFP or DsRed, and showed that CXCR4-tropic X4 and CCR5-tropic R5 viruses replicate differently in CD4$^+$ T cells simultaneously infected with X4 HIV-1 encoding EGFP and R5 HIV-1 encoding

DsRed (Yamamoto et al., 2009). Such recombinant HIV-1 strains encoding a fluorescent reporter gene will be even more valuable because of recent advances in multicolor flow cytometry, which permit more detailed characterization of HIV-1-infected cells.

Flow cytometry is a reliable and convenient method for analysis at the single cell level. Because the transcriptional activity of HIV-1 can be quantitatively monitored in indicator cells according to the fluorescence intensity of an EGFP reporter driven by the HIV-1 LTR (Dorsky et al., 1996; Gervaix et al., 1997; Kar-Roy et al., 2000), we investigated whether the HIV-1-expressing fluorescent reporters EGFP and DsRed would allow the quantitative evaluation of viral replication using a flow cytometer. The results show that a fluorescent reporter signal generated by recombinant HIV-1 strains enables the detection of infected cells at various stages of the viral life cycle.

MATERIALS AND METHODS

CELL PREPARATION

Human peripheral blood samples were collected from healthy donors after written informed consent. Sample collection was approved by the Institutional Ethical Committee of the National Institute of Infectious Diseases (NIID; Tokyo, Japan). Peripheral blood mononuclear cells (PBMCs) were separated on a Ficoll–Hypaque density gradient (Lymphosepal; IBL, Gunma, Japan) and $CD4^+$ T cells were negatively selected from the PBMCs using an EasySep Human $CD4^+$ T cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada).

CEM cells stably expressing human CCR5 (CEM–CCR5) were established by transducing CEM cells with the human *ccr5* gene using a conventional mouse retrovirus system. CEM–CCR5 cells were maintained in complete RPMI medium (10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) supplemented with 1 μg/ml puromycin at 37˚C.

PREPARATION OF HIV-1 VIRUS STOCKS

We previously constructed pNL432-based proviral clones encoding EGFP (pNL-E) or DsRed (pNL-D) for X4-tropic HIV-$1_{NL\text{-}E}$ or HIV-$1_{NL\text{-}D}$, respectively, and pNLAD8-based proviral clones encoding EGFP (pNLAD8-E) or DsRed (pNLAD8-D) for R5-tropic HIV-$1_{NLAD8\text{-}E}$ or HIV-$1_{NLAD8\text{-}D}$, respectively (Yamamoto et al., 2009; **Figure 1**). To prepare the HIV-1 viral stocks, the human embryonic kidney cell line 293T was transfected with pNL-E, pNL-D, pNLAD8-E, or pNLAD8-D using the calcium phosphate precipitation method and then incubated for 48 h. Culture supernatants were filtered and frozen at −80˚C. The amount of virus in each culture supernatant was measured using an in-house HIV-1 Gag p24 enzyme-linked immunosorbent assay (ELISA; Tsunetsugu-Yokota et al., 1995).

STIMULATION OF T CELL RECEPTORS

T cell receptors (TCR) were stimulated as described previously (Yamamoto et al., 2009) with some modifications. In brief, primary $CD4^+$ T cells were suspended in complete RPMI medium supplemented with 5% human plasma and stimulated with 5 μg/ml of immobilized anti-human CD3 monoclonal antibody (mAb; eBioscience, San Diego, CA) and 1 μg/ml of soluble anti-human CD28 mAb (eBioscience) in U-bottom, 96-well plates at 37˚C for 4 (weak stimulation) or 24 h (strong stimulation).

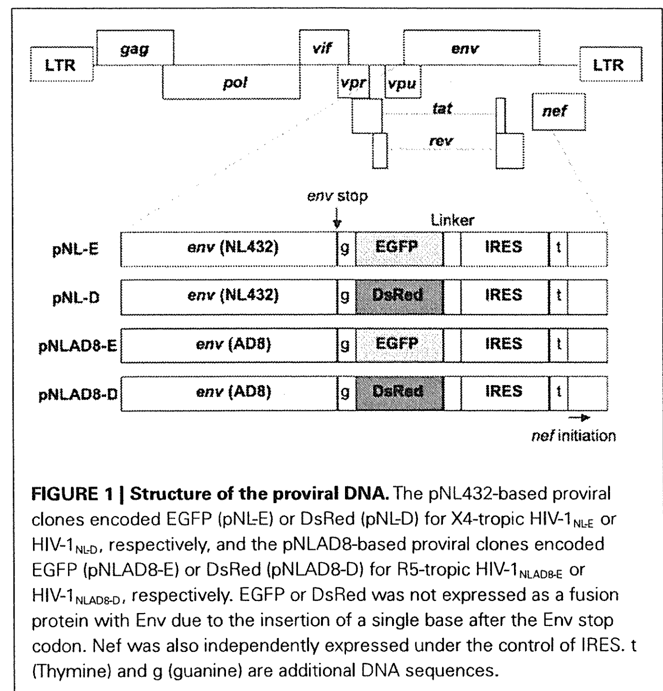


FIGURE 1 | Structure of the proviral DNA. The pNL432-based proviral clones encoded EGFP (pNL-E) or DsRed (pNL-D) for X4-tropic HIV-$1_{NL\text{-}E}$ or HIV-$1_{NL\text{-}D}$, respectively, and the pNLAD8-based proviral clones encoded EGFP (pNLAD8-E) or DsRed (pNLAD8-D) for R5-tropic HIV-$1_{NLAD8\text{-}E}$ or HIV-$1_{NLAD8\text{-}D}$, respectively. EGFP or DsRed was not expressed as a fusion protein with Env due to the insertion of a single base after the Env stop codon. Nef was also independently expressed under the control of IRES. t (Thymine) and g (guanine) are additional DNA sequences.

HIV-1 INFECTION AND CELL CULTURE

Primary $CD4^+$ T cells (either unstimulated or pre-TCR-stimulated) or CEM–CCR5 cells were infected with 200 ng of p24-measured amounts of HIV-$1_{NL\text{-}E}$, HIV-$1_{NL\text{-}D}$, HIV-$1_{NLAD8\text{-}E}$, or HIV-$1_{NLAD8\text{-}D}$ per 1×10^6 cells by spinoculation at $1200 \times g$ for 2 h at 25 (conventional conditions) or 4˚C (for CEM–CCR5 cells), as described previously (O'doherty et al., 2000; Dai et al., 2009). After spinoculation, cells were washed three times with PBS. Primary $CD4^+$ T cells were then suspended in complete RPMI medium supplemented with 5% human plasma. The cell suspensions derived from unstimulated or pre-TCR-stimulated $CD4^+$ T cells were settled onto U-bottom, 96-well plates with or without TCR-stimulation, respectively, at 37˚C for 24 h. After the 24 h culture, cells were washed three times with PBS, suspended in complete RPMI medium supplemented with 5% human plasma and 50 U/ml recombinant interleukin-2, and cultured in U-bottom, 96-well plates at 37˚C for up to 4 days.

FLOW CYTOMETRY

Cells were stained with fluorescence-conjugated mAbs as described previously (Yamamoto et al., 2009). The following mAbs were used for flow cytometry in various combinations: Pacific Blue-conjugated anti-human CD3 mAb (BioLegend, San Diego, CA, USA), phycoerythrin Cy7-conjugated anti-human CD4 mAb (BioLegend), and Alexa Fluor 700-conjugated anti-human CD8a mAb (BioLegend); and Nu24 mAb specific for HIV-1 Gag p24 (kindly provided by Dr. T. Sata, NIID, Tokyo, Japan) and conjugated to Alexa Fluor 647 using an Alexa Fluor 647 Protein Labeling Kit (Molecular Probes, Eugene, OR, USA). Dead cells were stained with propidium iodide or a LIVE/DEAD Fixable Dead Cell Stain

Kit (L34957; Invitrogen, Carlsbad, CA, USA). Intracellular staining (ICS) by Nu24 mAb was performed using a FIX and PERM Fixation and Permeabilization Kit (Invitrogen). Data collection was performed using a FACSCanto II (BD Bioscience, San Diego, CA, USA) and the data were analyzed using FACSDiva software (BD Bioscience) and FlowJo software (Tree Star, San Carlos, CA, USA).

QUANTIFICATION OF REPLICATED HIV-1 IN CELL CULTURE SUPERNATANTS

Human immunodeficiency virus type 1 replication was quantified in cell culture supernatants by ELISA and real-time RT-PCR. Gag p24 was measured using a RETRO-TEK HIV-1 p24 Antigen ELISA (ZeptoMetrix Corporation, Buffalo, NY, USA). For real-time RT-PCR, viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) and subjected to real-time RT-PCR using a SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen), a set of HIV-1 *gag*-targeted primers, and a TaqMan probe as previously described (Saito et al., 2010). PCR was performed in an Mx3000P (Stratagene, La Jolla, CA, USA).

RESULTS

CD4 DOWNMODULATION IS ASSOCIATED WITH HIV-1 FLUORESCENT REPORTER INTENSITY

The cell surface CD4 molecule is downmodulated in HIV-1-infected cells in response to the HIV-1 components Env, Nef, and Vpu (Malim and Emerman, 2008). Therefore, to investigate the correlation between the level of CD4 downmodulation and the HIV-1 fluorescent reporter intensity, primary CD4$^+$ T cells infected with HIV-1$_{NL-E}$, HIV-1$_{NL-D}$, HIV-1$_{NLAD8-E}$, or HIV-1$_{NLAD8-D}$ followed by TCR-stimulation for 1 day and cultivation for a further 4 days were analyzed by flow cytometry. As shown in **Figure 2** (left panels), HIV-1-infected cells expressing a fluorescent reporter signal, EGFP, or DsRed, were detected, although the numbers varied between individual donors ($n = 3$–4): about 10–30% for X4-tropic HIV-1$_{NL-E}$-infected and HIV-1$_{NL-D}$-infected cells and 1–10% for R5-tropic HIV-1$_{NLAD8-E}$-infected and HIV-1$_{NLAD8-D}$-infected cells. However, the number of HIV-1$^+$ cells was comparable between HIV-1$_{NL-E}$ and HIV-1$_{NL-D}$ (X4-tropic), and between HIV-1$_{NLAD8-E}$ and HIV-1$_{NLAD8-D}$ (R5-tropic) within each donor, showing that the fluorescent reporter genes encoded within the HIV-1 proviral genome did not affect HIV-1 infectivity as described previously (Yamamoto et al., 2009). When we categorized CD3$^+$CD8$^-$ T cells into three fractions (HIV-1-negative, -dull, and -high) based on the fluorescence intensity of EGFP and DsRed, we found that CD4 was strongly downmodulated in the HIV-1 high fraction in all the HIV-1 strains (**Figure 2**, right panels). Interestingly, CD4 was also downmodulated in the HIV-1 dull fraction, but the level was modest compared with that in the HIV-1 high fraction (**Figure 2**, right panels). These results indicate that the level of CD4 downmodulation is associated with HIV-1 fluorescent reporter intensity.

FIXATION/PERMEABILIZATION WEAKENS THE HIV-1 FLUORESCENT REPORTER SIGNAL

To investigate the correlation between HIV-1 fluorescent reporter intensity and viral replication levels, we attempted to perform ICS for Gag p24 in HIV-1-infected cells prepared as described above. When we observed X4-tropic HIV-1$_{NL-E}$-infected and HIV-1$_{NL-D}$-infected cells from three donors by flow cytometry, we noticed that fixation/permeabilization, an essential step for ICS, weakened the fluorescent reporter signal for both EGFP and DsRed. **Figure 3** shows the flow cytometry profiles obtained for EGFP and DsRed at identical photomultiplier tube (PMT) voltages between intact (untreated) cells and fixed/permeabilized cells to visualize the differences in fluorescent reporter intensity. DsRed$^+$ cells were not properly separated from DsRed$^-$ cells within the population treated by fixation/permeabilization; the frequency of DsRed$^+$ cells was, therefore, markedly decreased. No adjustment of the flow cytometer settings, including PMT voltage and compensation, improved the blunted fluorescent reporter signal generated after fixation/permeabilization. Nevertheless, the number of EGFP$^+$ cells within the intact cell and fixed/permeabilized cell populations was comparable. Similar results were obtained for R5-tropic HIV-1$_{NLAD8-E}$ and HIV-1$_{NLAD8-D}$ (data not shown). Taken together, these results indicate that it is preferable to use an EGFP reporter when the fixation/permeabilization of cells is required.

HIV-1 FLUORESCENT REPORTER SIGNALS RELIABLY DETECT PRODUCTIVELY INFECTED CELLS SHOWING DIFFERENT VIRAL REPLICATION LEVELS

Following the results shown in **Figure 3**, we next assessed viral replication levels in the HIV-1$_{NL-E}$ infection group (5 days culture) from six donors using Gag p24 ICS (**Figure 4**). A representative flow cytometric analysis showed that not all EGFP$^+$ cells were Gag$^+$ and *vice versa*. When CD4 expression levels were compared in each of the four cell fractions based on the expression patterns of EGFP and Gag p24 (EGFP$^+$Gag$^+$, EGFP$^+$Gag$^-$, EGFP$^-$Gag$^+$, and EGFP$^-$Gag$^-$), the strongest downmodulation of CD4 was observed in EGFP$^+$Gag$^+$ cells (red fraction). CD4 downmodulation was moderate in EGFP$^+$Gag$^-$ cells (green fraction). However, CD4 was not downmodulated at all in EGFP$^-$Gag$^+$ cells (blue fraction) and the expression level of CD4 was the same as that in EGFP$^-$Gag$^-$ cells (black fraction). We further divided the EGFP$^+$Gag$^+$ cells (red fraction) into Gag^{hi} (brown fraction) and Gag^{lo} cells (pink fraction) and compared the expression levels of EGFP and CD4 with those of Gag p24. Gag^{hi} cells (brown fraction) showed the strongest expression of EGFP and the strongest downmodulation of CD4. Gag^{lo} cells (pink fraction) showed an intermediate level of EGFP expression [between that of Gag^{hi} cells (brown fraction) and that of EGFP$^+$Gag$^-$ cells (green fraction)] and CD4 expression [between that of Gag^{hi} cells (brown fraction) and EGFP$^-$Gag$^-$ cells (black fraction)]. These results indicate that the expression level of EGFP correlates with that of Gag p24 in HIV-1-infected cells in which CD4 is downmodulated.

HIV-1-BOUND OR -INTERNALIZED CELLS ARE ALSO DETECTED BY Gag p24 ICS

Because CD4 downmodulation was not observed in EGFP$^-$Gag$^+$ cells (**Figure 4**; blue fraction), it is possible that these cells may still be bound by or have internalized HIV-1 but have not produced virions. Therefore, we next investigated the kinetics of EGFP$^-$Gag$^+$ cells during 5 days post-infection. Primary CD4$^+$

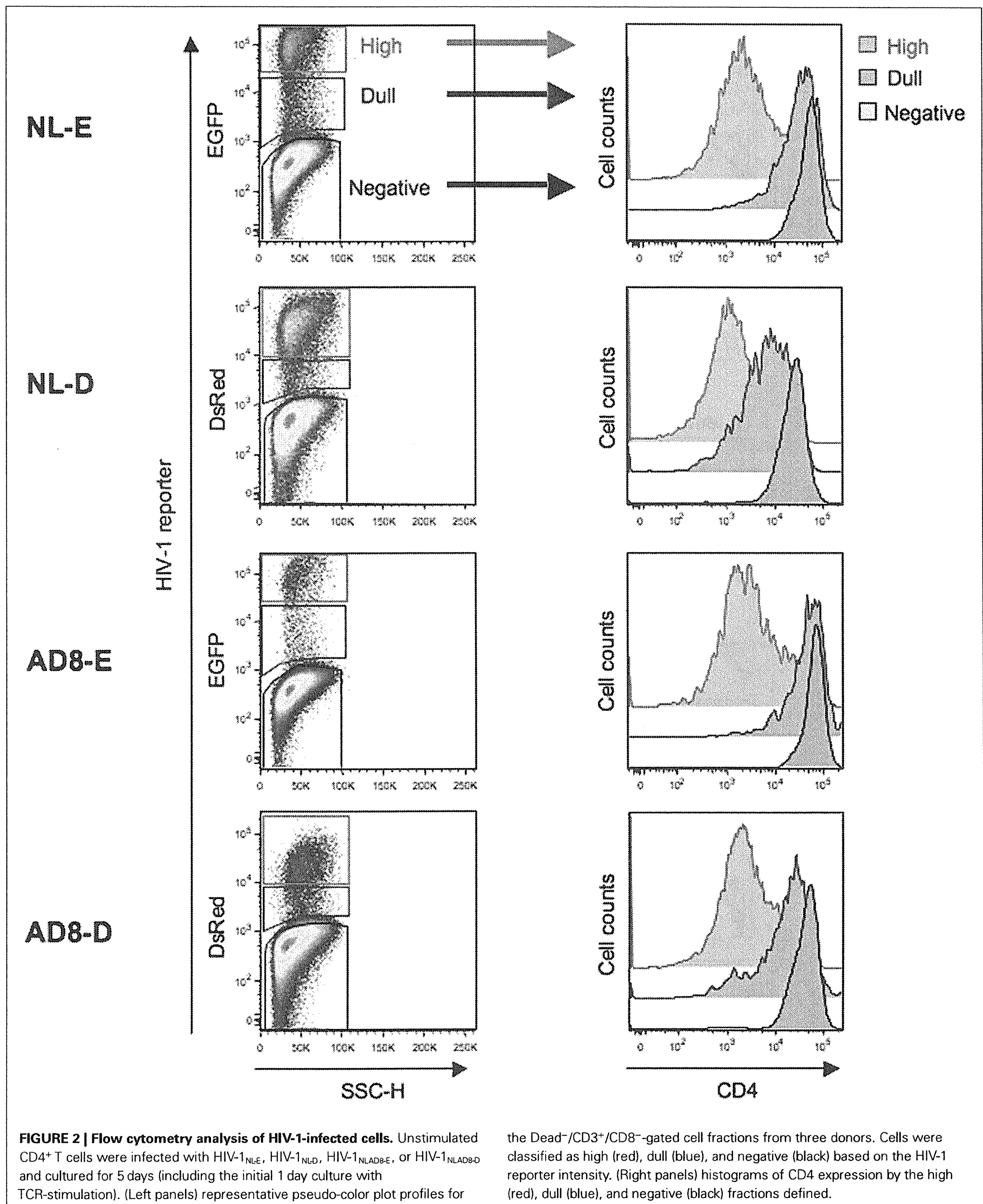


FIGURE 2 | Flow cytometry analysis of HIV-1-infected cells. Unstimulated $CD4^+$ T cells were infected with HIV-1_{NL-E}, HIV-1_{NL-D}, HIV-$1_{NLAD8-E}$, or HIV-$1_{NLAD8-D}$ and cultured for 5 days (including the initial 1 day culture with TCR-stimulation). (Left panels) representative pseudo-color plot profiles for the $Dead^-/CD3^+/CD8^-$-gated cell fractions from three donors. Cells were classified as high (red), dull (blue), and negative (black) based on the HIV-1 reporter intensity. (Right panels) histograms of CD4 expression by the high (red), dull (blue), and negative (black) fractions defined.

T cells from three donors were infected with HIV-1_{NL-E} followed by TCR-stimulation for 1 day and cultivation for a further 4 days. **Figure 5A** shows a representative flow cytometric analysis. At 1 day post-infection, 17.6% of Gag $p24^+$ cells were observed,

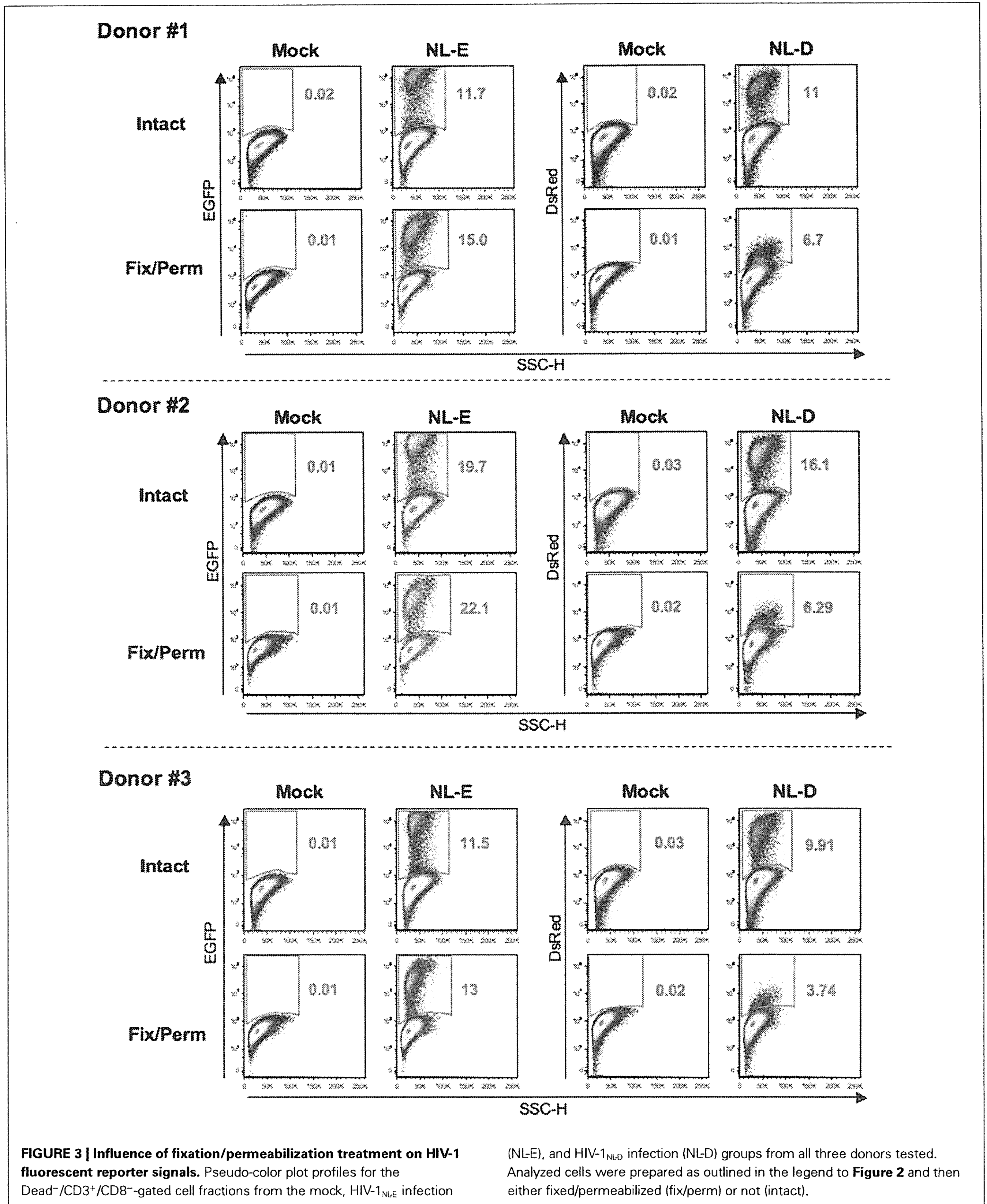


FIGURE 3 | Influence of fixation/permeabilization treatment on HIV-1 fluorescent reporter signals. Pseudo-color plot profiles for the Dead$^-$/CD3$^+$/CD8$^-$-gated cell fractions from the mock, HIV-1$_{NL-E}$ infection (NL-E), and HIV-1$_{NL-D}$ infection (NL-D) groups from all three donors tested. Analyzed cells were prepared as outlined in the legend to **Figure 2** and then either fixed/permeabilized (fix/perm) or not (intact).

despite the fact that no EGFP$^+$ cells were detected. At 2 days post-infection, the proportion of EGFP$^-$Gag$^+$ cells was decreased and EGFP$^+$ cells including Gag p24$^+$ and Gag p24$^-$ cells became to be observed, suggesting that initially infecting HIV-1 was

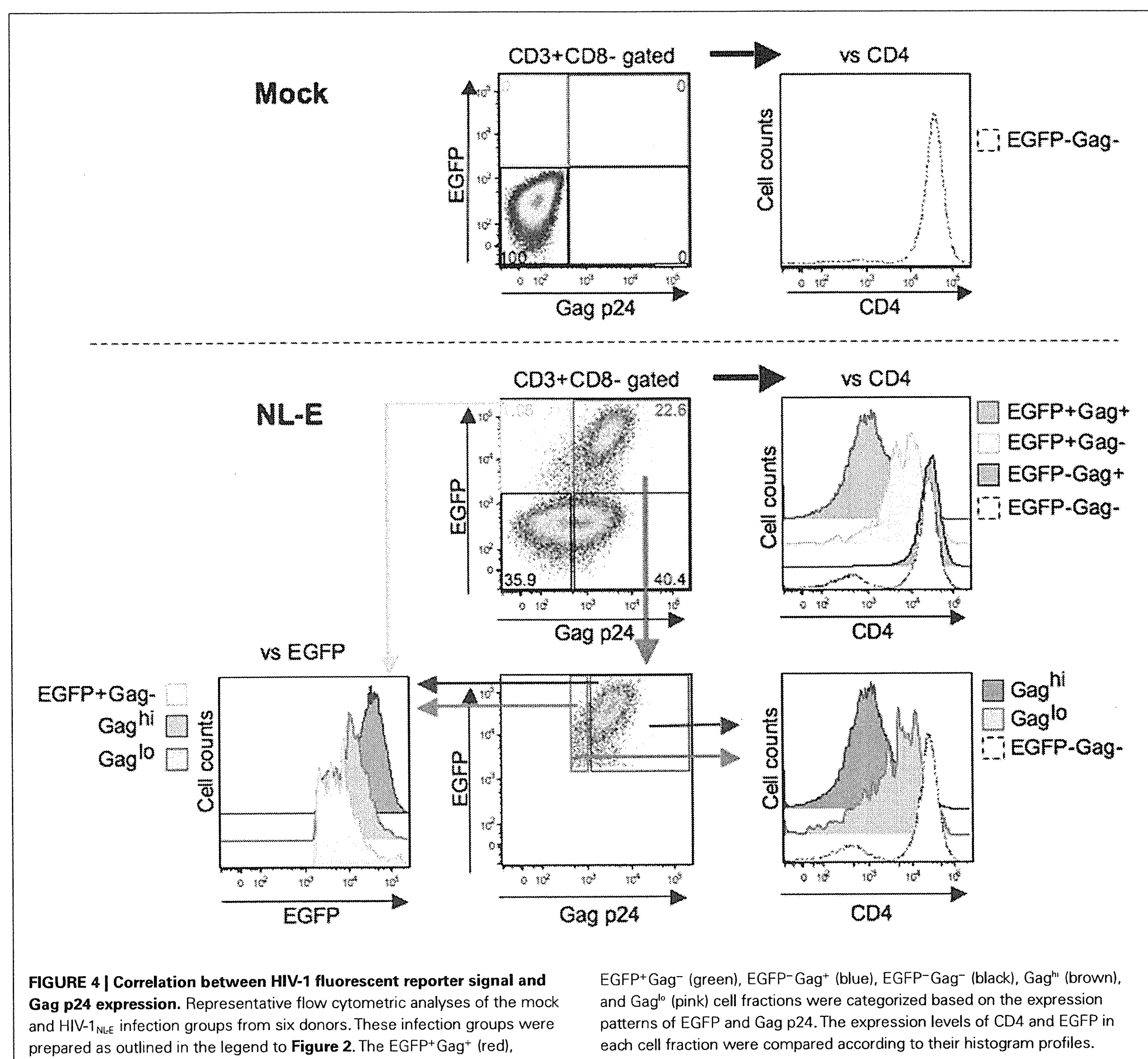


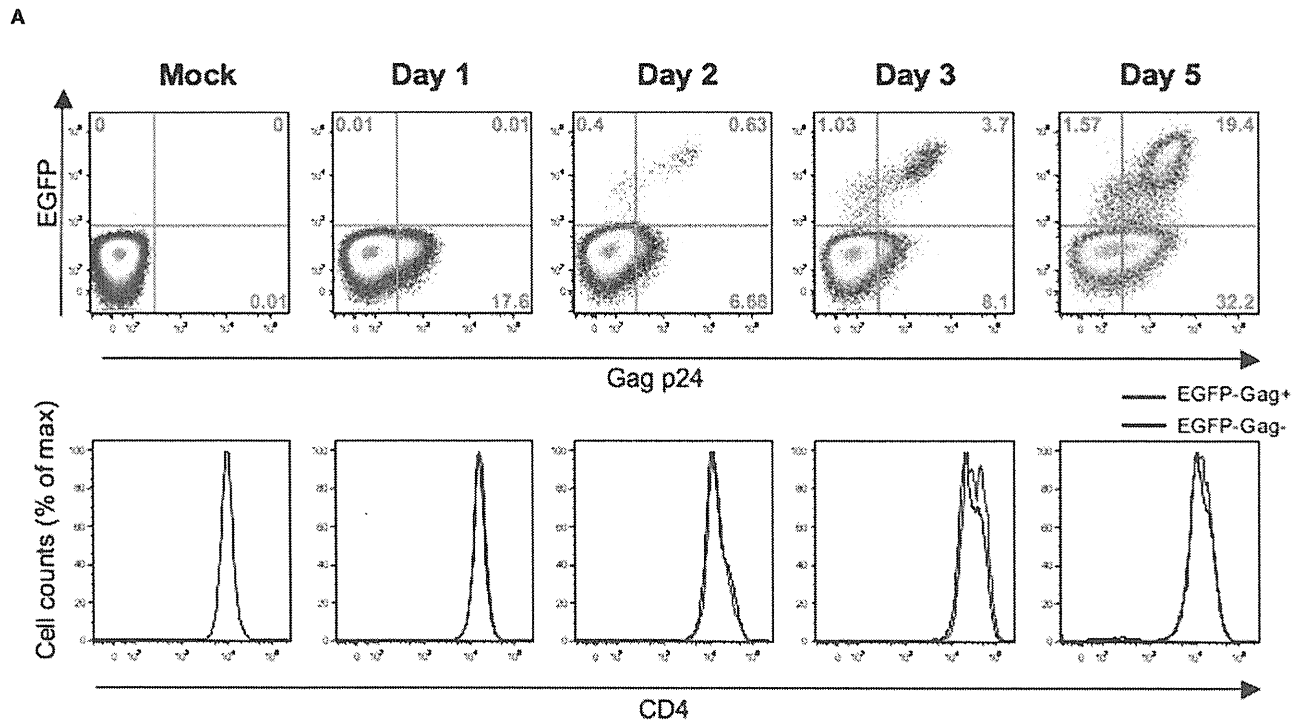
FIGURE 4 | Correlation between HIV-1 fluorescent reporter signal and Gag p24 expression. Representative flow cytometric analyses of the mock and HIV-1_{NL-E} infection groups from six donors. These infection groups were prepared as outlined in the legend to **Figure 2**. The $EGFP^+Gag^+$ (red), $EGFP^+Gag^-$ (green), $EGFP^-Gag^+$ (blue), $EGFP^-Gag^-$ (black), Gag^{hi} (brown), and Gag^{lo} (pink) cell fractions were categorized based on the expression patterns of EGFP and Gag p24. The expression levels of CD4 and EGFP in each cell fraction were compared according to their histogram profiles.

degraded and/or replaced with replication-competent proviruses. After 3 days post-infection, $EGFP^+$ cells were clearly visible and the proportion of $EGFP^-Gag^+$ cells turned to be increased, suggesting that progeny virus infection occurred. Because the CD4 expression levels were identical between $EGFP^-Gag^+$ cells and $EGFP^-Gag^-$ cells throughout the culture period, Gag p24 ICS must have detected cells that had bound or internalized HIV-1.

CEM–CCR5 cells, which are almost as susceptible to X4 and R5 HIV-1 fusion (data not shown), were used to confirm that Gag p24 ICS did indeed detect HIV-1-bound cells. Also, because it has been suggested that spinoculation at 25°C may induce HIV-1 fusion to the targeted cells (Dai et al., 2009), we tested Gag p24 ICS using CEM–CCR5 cells immediately after spinoculation with X4-tropic HIV-1_{NL-E} or R5-tropic HIV-1_{AD8-E} at 4°C. When cells were not fixed/permeabilized, no Gag $p24^+$ cells were detected by flow cytometry (**Figure 5B**, upper panels); however, when cells were fixed/permeabilized, a substantial proportion of Gag^+ cells was detectable in both the HIV-1_{NL-E} and HIV-1_{AD8-E} infection groups (**Figure 5B**, lower panels). Taken together, these results indicate that cells that have bound or internalized HIV-1 can be detected using flow cytometry for Gag p24 ICS.

THE INTENSITY OF THE HIV-1 FLUORESCENT REPORTER SIGNAL DEPENDS ON TCR-MEDIATED ACTIVATION LEVELS

T cell receptors-mediated activation of HIV-1-infected $CD4^+$ T cells increased productive viral replication, although the signaling pathway responsible may be different for X4 and R5 HIV-1 (Popik and Pitha, 2000). We investigated whether the intensity of the HIV-1 fluorescent reporter signal was affected by TCR-mediated



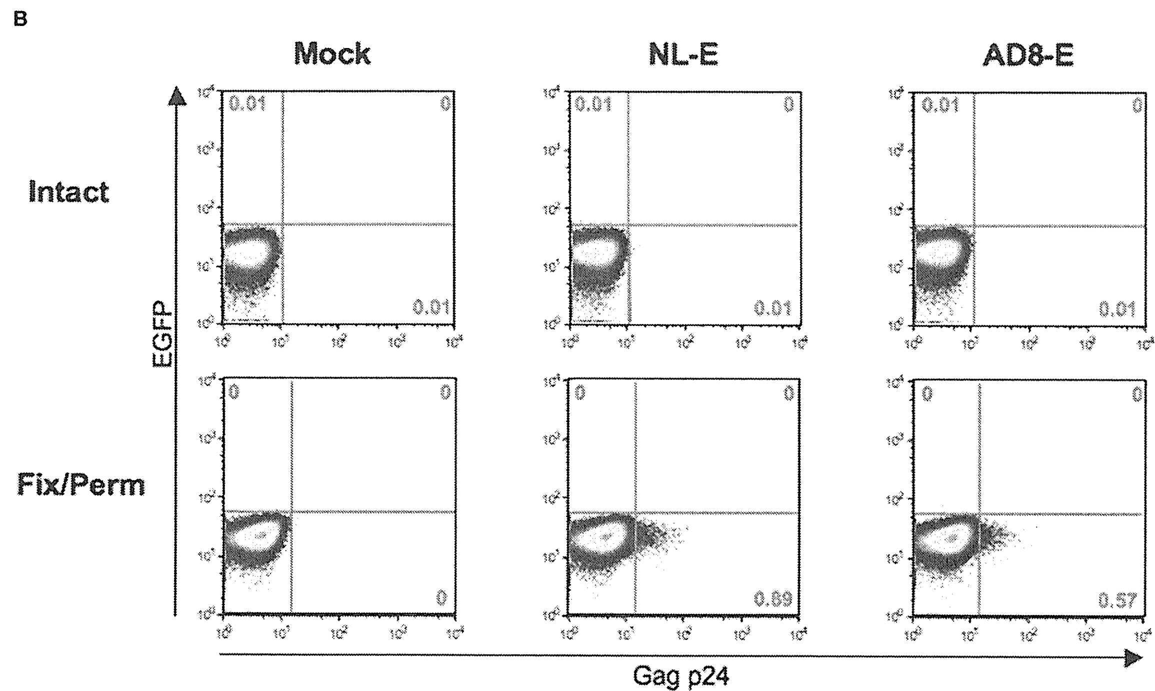


FIGURE 5 | Evaluation of Gag p24 ICS for HIV-1-internalized and -bound cells. (A) Representative flow cytometric analyses of the HIV-1_{NL-E} infection (NL-E) and mock (Mock) groups from three donors in which unstimulated primary $CD4^+$ T cells were infected with HIV-1_{NL-E} or not, respectively, and cultured for 5 days (including the initial 1 day culture with TCR-stimulation). The expression patterns of EGFP and Gag p24 are indicated in the pseudo-color plot profiles for the $Dead^-/CD3^+/CD8^-$-gated cell fractions (upper panels). The expression level of CD4 in $EGFP^-Gag^+$ and $EGFP^-Gag^-$ cells is indicated in the histograms by the blue and black lines, respectively (lower panels). **(B)** Representative flow cytometric analyses of Gag p24 staining. CEM–CCR5 cells were infected with HIV-1_{NL-E} (NL-E), HIV-1_{AD8-E} (AD8-E), or not (Mock). Immediately after spinoculation at 4°C, the cells were washed and fixed/permeabilized (fix/perm) or not (intact) prior to Gag p24 staining.

activation levels. In this experiment, primary CD4$^+$ T cells from four donors were individually pre-stimulated via the TCR for 4 (weak stimulation) or 24 h (strong stimulation), infected with HIV-1_{NL-E}, and then cultured for a further 3 days. First, we confirmed that this experimental protocol allowed the preferential production of HIV-1_{NL-E} upon strong stimulation in all donors by examining the cell culture supernatants by ELISA (**Figure 6A**) and real-time RT-PCR (**Figure 6B**). Flow cytometric analysis of intact cells showed that HIV-$1_{NL-E}$$^+$ (EGFP$^+$) cells were more prevalent after strong stimulation than after weak stimulation, although the proportion of HIV-$1_{NL-E}$$^+$ cells varied among individuals (**Figure 6C**, upper and middle panels). The PMT voltage was optimized for EGFP to prevent excessive EGFP signaling (**Figures 2** and **3**). Of note, EGFP expression by HIV-$1_{NL-E}$$^+$ cells was lower in the weak stimulation group than in the strong stimulation group (as observed in donors #4 and #5), and EGFP expression in the weak stimulation group approached that in the strong stimulation group in parallel with the increase in the number of HIV-$1_{NL-E}$$^+$ cells (as observed in donors #6 and #7; **Figure 6C**, lower panels). Taken together, these results show that the intensity of the fluorescent reporter is highly correlated with the viral replication level.

DISCUSSION

Flow cytometric analysis is a reliable and convenient method for detecting HIV-1-infected cells at a single cell level. Here, we studied the potential usefulness of several HIV-1 fluorescent reporters that have been published previously (Yamamoto et al., 2009). We examined whether they would be helpful for evaluating viral replication levels based on their fluorescence intensity. In this study, we used recombinant HIV-1 encoding either EGFP or DsRed to show that the fluorescence intensity of the EGFP and DsRed reporters was associated with the level of CD4 downmodulation (**Figure 2**). Furthermore, we showed that EGFP intensity was associated with the expression level of Gag p24 (**Figure 4**). These findings clearly indicate that fluorescent reporter intensity is useful for evaluating viral replication levels. To confirm this argument, we further compared the fluorescent reporter intensity of HIV-1-infected cells that were strongly or weakly stimulated via the TCR. As expected, higher levels of HIV-1 replication/production occurred in strongly stimulated cells from all the donors tested (**Figure 6A,B**). Although the proportion and EGFP intensity of the HIV-1-infected cells varied among individuals, this might be due to differing susceptibility to HIV-1 and/or TCR-stimulation. Thus, the variability in EGFP expression is rather favorable to our argument, as increased EGFP intensity was associated with an increase in the number of HIV-1-infected cells after weak stimulation (**Figure 6C**).

Although Gag p24 ICS is usually used for flow cytometric analysis of other markers, we showed that it can also be used to detect cells that have internalized or bound HIV-1 (**Figure 5A,B**). However, Gag p24 ICS did not appear sensitive enough to detect HIV-1-infected cells because some HIV-1-infected cells in which CD4 was moderately downmodulated were identified as positive for EGFP but negative for Gag p24 (**Figure 4**). Bosque and Planelles (2009) also identified a small population of such reporter-positive but Gag p24-negative cells by flow cytometry when CD4$^+$ T cells were infected with EGFP-encoded DHIV incorporating a small out-of-frame deletion in the gp120-encoding area and pseudotyped with X4-tropic HIV-1_{LAI}, and assumed that these cells were at an early stage of the infection process and did not display late viral proteins. Therefore, our own findings indicate that it is the HIV-1 fluorescent reporter, rather than Gag p24 staining, that reliably detects HIV-1-infected cells at different stages of infection in flow cytometry experiments.

It is known that maturation of DsRed for coloration is usually slower compared with EGFP (Bevis and Glick, 2002; Maruyama et al., 2004). When we focused on the HIV-1 dull fraction in **Figure 2**, we found that CD4 downmodulation was stronger in DsRed$^+$ cells than in EGFP$^+$ cells. These results suggest that the EGFP reporter is preferable to the DsRed reporter for detection of earlier stage of infection. Furthermore, the detrimental effect of fixation/permeabilization on fluorescent reporter intensity, particularly when using the DsRed reporter, should be noted (**Figure 3**). Although the detailed mechanism remains obscure, this may result from the lower fluorescence intensity of DsRed compared with EGFP. A similar phenomenon was described regarding fixation with 3% paraformaldehyde, which significantly decreases the fluorescence intensity of DsRed, although specific data were not provided (Weber et al., 2006). Regardless of the weakened signal, the EGFP reporter is still compatible with fixation/permeabilization because the proportion of EGFP$^+$ cells was comparable between intact cells and fixated/permeabilized cells (**Figure 3**). Therefore, the EGFP reporter still maintains an advantage for analyses of cytokine/chemokine production and proliferation assays based on Ki-67 expression, for which ICS is necessary.

The HIV-1 fluorescent reporter has a potential application in molecular biology. In general, ICS-treated cells are not suitable for analysis using molecular biology techniques, since formaldehyde-based fixation (required for ICS) makes RNA extraction and reverse transcription and quantification problematic (Farragher et al., 2008) because of chemical cross-linking of proteins and nucleic acids (Kuykendall and Bogdanffy, 1992; Finke et al., 1993; Park et al., 1996), degradation of RNA (Bresters et al., 1994), and covalent modification of RNA via the addition of monomethylol groups to the bases (Masuda et al., 1999); therefore, by using the HIV-1 fluorescent reporter, HIV-1-infected cells can be sorted/purified without the need for fixation, allowing further characterization at a molecular level.

Given the usefulness of the HIV-1 fluorescent reporter shown here, it would also be very useful for investigating the mechanisms involved in the selective replication of R5 HIV-1 over X4 HIV-1 during the acute phase *in vivo* (Wolinsky et al., 1992; Zhu et al., 1993; van't Wout et al., 1994) and in cell culture systems *in vitro* (Schweighardt et al., 2004; Roy et al., 2005). We previously developed an *in vitro* dual infection model using EGFP-encoded X4 HIV-1 (HIV-1_{NL-E}) and DsRed-encoded R5 HIV-1 (HIV-1_{AD8-D}) and showed that the increase in the proportion of X4 HIV-1-infected cells is dependent upon their activation level (Yamamoto et al., 2009). Furthermore, the results of the present study show that the fluorescence intensity of the reporter molecule